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RESEARCHES ON THE NATURE OF ENZYME-ACTION.

I. On the causes of the Rise in electrical conductivity under the action of Trypsin.

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Introductory. The first investigator who made experimental use of the change in electrical conductivity shown by various solutions under the action of enzymes was Sjögvist[1]. He found that the hydrolysis of albumins by pepsin was accompanied by a diminution of conductivity. This fact was confirmed by Oker-Blom[2], who also observed that the action of trypsin was accompanied by a rise in conductivity and advocated the method as of great convenience in the study of the action of various proteolytic enzymes. It has been employed for this purpose by Victor Henri and Larguier des Bancels[3], who have published a few experiments chiefly on the law of action of trypsin on gelatin.

I have myself carried out a large number of experiments for the most part on trypsin, a preliminary account of which will be found in the *Arch. des Sciences Biologiques*, XI. Supp. p. 261, St Petersburg, 1904.

[1] *Skand. Arch. f. Physiol.* v. p. 364. 1895.

[2] *Versamm. Nordischer Naturf. in Helsingfors, Verh. d. Sektion f. Anat. u. Physiol.* p. 9. 1902.

[3] *C. R. Société de Biol.* pp. 563 and 787. 1903.

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The chief advantage which this method presents and which it shares in common with other physical methods, such as the measurement of optical activity, is the rapidity with which a large number of determinations can be made, so that it is possible to follow closely the course of a reaction even if this reaction proceeds at a considerable velocity. It also renders possible, without the expenditure of an undue amount of labour, the study of the effects of the numerous factors which affect the rate of change. By methods of chemical analysis these investigations would necessitate a very large amount of time.

This being so, it is obviously a matter of some interest and practical importance to know how far the change in electrical conductivity can be taken as representative of the action of the enzyme.

In my previous paper[1] I have given some indications as to the possible causes of the change of conductivity in the case of trypsin, and in the present paper I hope to throw further light on the subject by the discussion of some recent experimental results.

It is necessary to bear in mind that the passage of an electrical current through a solution is rendered possible by the presence therein of ions, each of which carries a definite charge of electricity from one electrode to the other. The rate of movement of these ions depends upon various conditions, such as temperature, presence of non-electrolytes, and so on. It is obvious, therefore, that an increase in the quantity of electricity passing in unit time, or, in other words, in the electrical conductivity, may be brought about, other conditions remaining constant, either by an increase in the velocity of the migration of the ions, or by increasing their number; and a decrease of conductivity may be produced by changes of the opposite kind.

When, therefore, we find that a solution of a protein or similar body undergoes an increase in conductivity when acted on by trypsin, the explanation may be, either that the diminution of internal friction, which we know takes place, allows the ions already present to move at a greater rate; or on the other hand there may be an actual increase in the number of ions present, owing to a separation of electrolytes from the protein molecule. Both of these causes may be active at the same time.

[1] *Loc. cit.* p. 14 of reprint.

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Changes in Viscosity.

It is easy to show that the first cause, although it may assist to a slight degree, is altogether inadequate to explain the whole of the effect produced by trypsin.

In the first place the time course of the change of internal friction is very different from that of the electrical conductivity. In the curves given in my former paper (p. 15 of the reprint) it will be seen that the internal friction diminishes much faster than the conductivity increases, and also that the former change has come to an end while the conductivity is still increasing. Owing to the extremely rapid change in the internal friction under the action of trypsin at 38° I have repeated the experiment at a temperature of 19°·3.

Exp. 40 c.c. of a 5 % solution of caseinogen in ammonia, acid to phenol-phthalein, alkaline to litmus, warmed in flask in thermostat at 19°·3. 5 c.c. of 2 % filtered watery extract of "Rhenania" pancreatin, also previously warmed, added, shaken, and portions immediately poured into a conductivity vessel and an Ostwald viscosimeter already immersed in the thermostat. Resistance and internal friction taken at intervals as follows:

Resistance values are given instead of specific conductivity since the changes of resistance and internal friction are in the same direction and therefore easier to compare.

Time in minutes	Rate of flow through capillary in seconds	Resistance in ohms
0	(183)*	(333)*
4	—	325·5
8	160	—
12	—	308·2
16	137	—
20	—	298·4
30	115	286·1
45	105	273·1
74	95	254·6
81	92	—
131	85	230·0
180	82	216·5
246	79	204·9
342	77	195·0
466	77	187·4
541	76	184·9
711	74	180·1

* Calculated. The change in viscosity at first is too rapid to admit of accurate measurement directly.

The curves of Fig. 1 are drawn from these values. The numbers on the left refer to the viscosity curve and give the values of its ordinates in seconds; those on the right refer to the electrical resistance curve and

give the values of its ordinates in ohms. In order to render the forms of the two curves more readily comparable I have drawn them on a scale such that the two curves meet at the end of the figure.

If we look also at the numerical values we shall see that at 131′, for example, the internal friction had diminished by $\frac{98}{109} = 90\,\%$ of the value attained in 12 hours, at the same moment of time the resistance had diminished by $\frac{103}{153} = 67\,\%$.

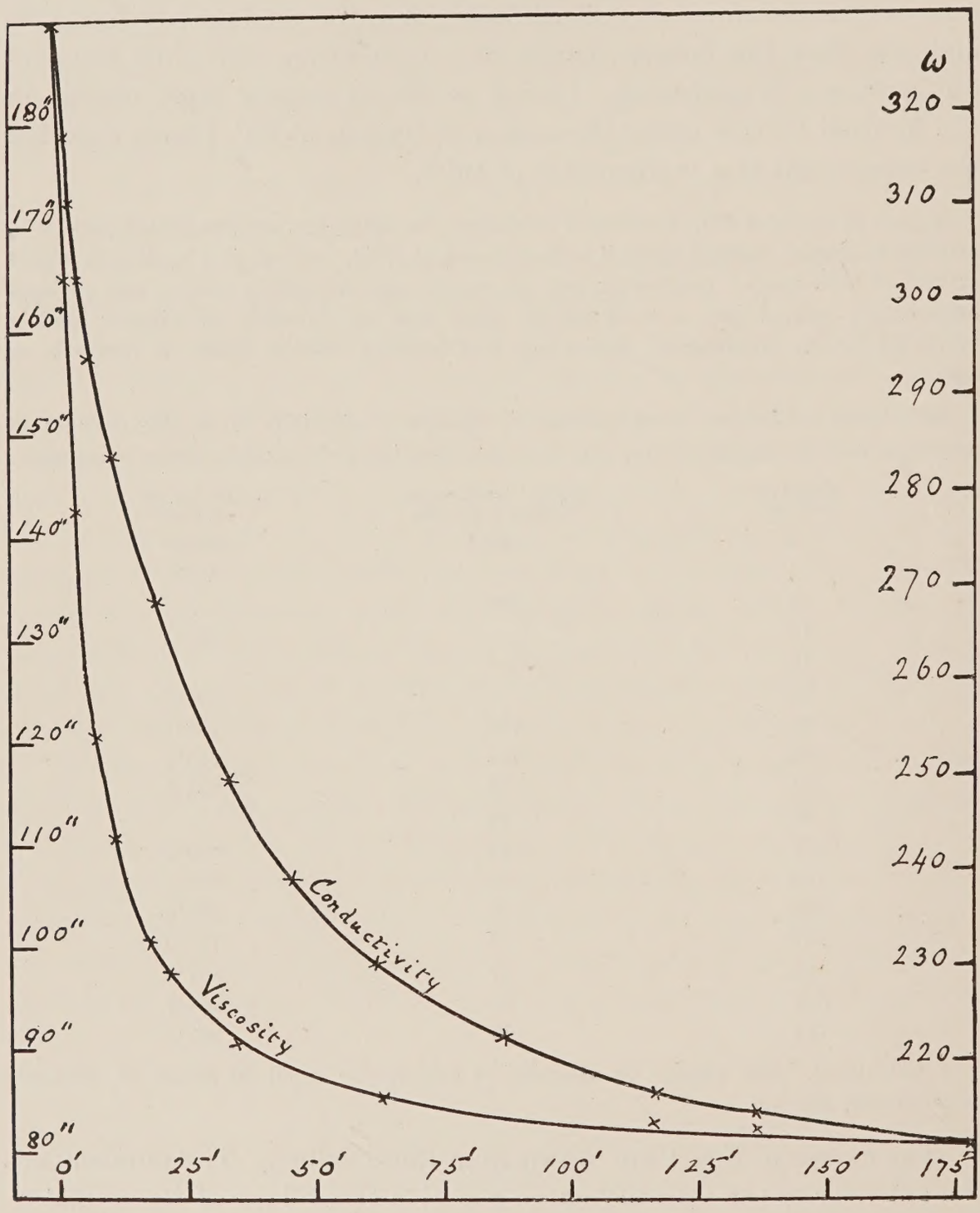

Fig. 1.

This rapid change in the internal friction is also very obvious if one takes a bottle of 10 % caseinogen solution which has been warmed to 40°, adds trypsin and shakes. It is seen that the viscid solution becomes quite limpid even while one shakes the bottle, that is in less than a minute.

Gelatin also, under the action of trypsin at 40°, very rapidly loses its power of solidifying on cooling. The following experiment shows this fact:—

Exp. 50 c.c. of a 5 % solution of gelatin, added 5 c.c. $\frac{n}{2}$ ammonia and warmed to 40°. When warm 5 c.c. of 2 % trypsin solution added, samples taken out at intervals and dropped on to a metal box filled with a mixture of ammonium nitrate and water of which the temperature was 8° C. The gelatin solidified rapidly at this temperature in the case of the samples up to 3 minutes after addition of trypsin, at 4 minutes the jelly was just to be detected by touching with the finger-tip, and at 5 minutes no jelly was formed until after standing for 20 minutes and then it was of the very weakest consistency.

Again, when solid fibrin is acted on by trypsin in alkaline solution at 40°, it is well known that it is rapidly taken into solution, but if, as soon as the solid has disappeared, the solution is heated to 100° it will be found that it has only been attacked so far as to convert it into a soluble coagulable protein.

Taking these facts into consideration, it seems to me that the methods of investigating proteolytic enzymes dependent upon mere changes of physical state give somewhat limited information as to the really important part of the work of these enzymes, viz. the chemical changes. The methods I refer to are such as those dependent on the liquefaction of solid gelatin solution at room temperature in Fermi's method, or those dependent on the solution of fibrin such as Grützner's and Vernon's.

In another way it can be shown that changes of internal friction are insufficient to account for the change in conductivity in the case of trypsin action.

A considerable amount of work has been done, chiefly by Arrhenius[1], on the diminution of conductivity produced by the addition of non-electrolytes to solution of electrolytes, but there is not much known as to the behaviour of colloidal non-conductors, such as concern us in the present case, when these are present in solutions of electrolytes. Accordingly I have thought it advisable to make a few experiments on this subject.

[1] *Zeitsch. f. physik. Chem.* IX. p. 493. 1892.

As regards gelatin in the first place:

An approximate $\frac{n}{50}$ solution of KCl was found to have a specific conductivity at 10° C. of 1990 gemmhos (= recip. megohms).

A portion of the same solution was used to make a 5 % solution of gelatin; the conductivity of this solution was 2100 gemmhos. That is, the addition of gelatin caused a slight *increase* in the conductivity. This was found to be accounted for by the conductivity of the gelatin itself, so that, as far as this experiment is concerned, the addition of gelatin had no perceptible effect on the conductivity of the KCl solution. This absence of any considerable effect of certain colloids on conductivity is, I believe, well known. It is, however, usually regarded as a peculiarity of the "gel" condition, and is, no doubt, connected with the facts observed by Reformatsky[1] and by Levi[2] with respect to the non-effect of gelatin, agar-agar, or colloidal silica on the velocity of certain reactions. In point of fact it is not necessarily connected with the "gel" condition, since it is shown also by gelatin solution at 30° as the following experiment demonstrates.

Exp. A quantity of gelatin (about 100 grams) was soaked in repeated changes of distilled water at room temperature. Solutions of various concentration of this gelatin were made in distilled water and in $\frac{n}{20}$ KCl. To compare with these, similar solutions of cane-sugar were made. It was found that, allowing for the conductivity of the gelatin itself, due to the adsorbed inorganic ash constituents, which were not completely washed out, the diminution in conductivity produced by the addition of equal quantities of gelatin or cane-sugar to a solution of potassium chloride was very nearly the same. It amounted to 2·8 % of the initial conductivity for each 1 % addition of cane-sugar and 3·4 % for gelatin, at a temperature of 38° C.

This experiment shows, then, that gelatin has about the same effect on the conductivity of electrolytes as has a corresponding amount of a non-colloidal non-conductor like sugar. The considerable viscosity of gelatin solutions, as measured by their rate of flow through narrow tubes, does not then show itself in any corresponding effect on the velocity of ionic migration. Its action in this respect is, in fact, of the same order as that of non-colloidal non-conductors, viz. from 1·5 to 4 %, as found by Arrhenius.

Moreover, when a gelatin solution is gradually warmed, so that it passes from the "gel" to the "sol" state and the conductivity determined at frequent intervals, there is found to be no inflection in the

[1] *Zeitsch. f. physik. Chem.* VII. p. 34. 1891.

[2] *Chem. Centralbl.* II. p. 658. 1900.

curve drawn from the values obtained. This would, of course, be the case if there were any difference between the "sol" and "gel" state in their action on electrical conductivity.

Caseinogen solutions, also, behave like ordinary non-electrolytes, as shown by the following experiments:

The specific conductivity of a mixture of equal parts of a 2 % caseinogen solution and $\frac{n}{10}$ KCl was, at 38°, 8400 gemmhos. That of $\frac{n}{20}$ KCl being 7300, and that of a mixture of equal parts of the caseinogen solution and water being 1580, the sum would be 8880 gemmhos; so that the mixture had a conductivity less by 480 gemmhos than that of the sum of its constituents. That is, the addition of 1 % of caseinogen to an electrolyte solution diminishes the conductivity of the electrolyte solution by $\frac{480}{8880} \times \frac{1}{2} = 2{\cdot}7$ %.

That gelatin is not entirely without effect on diffusion processes has been shown by Kurt Meyer[1] and by Bechhold and Ziegler[2] in work published since the completion of the above experiments.

At the same time it is quite evident that, even supposing all non-electrolytes to disappear during the action of trypsin, no greater effect than an increase of some 4 % of the original conductivity for each 1 % of the non-electrolyte could be obtained. In actual fact the increase produced by the action of trypsin amounts to 100 % or more.

It appears, indeed, that the effect of trypsin is to cause the production of bodies which have a somewhat less action in diminishing the conductivity than that of the original gelatin:

Exp. A 5 % solution of gelatin was taken, trypsin added and the mixture at once divided into two parts, one of which was heated to 100° and the other allowed to digest at 40° for 24 hours. 10 c.c. of each were then placed in conductivity vessels in the thermostat at 40° and the specific conductivities found to be:

Undigested	...	...	882 gemmhos
Digested	...	...	1434 ,,

1 c.c. of $\frac{n}{10}$ KCl was now added to each and the conductivity thereby raised to:

Undigested	...	...	2060 gemmhos
Digested	...	...	2700 ,,

that is, an increase of:

In the undigested solution	...	...	1178 gemmhos
In the digested solution	...	...	1266 ,,

or a difference of 7 % in favour of the digested solution.

In other similar experiments a less difference than this was found, but in any case it will be seen that the difference between the two solutions was quite inadequate to account for the actual increase of

[1] Hofmeister's *Beiträge*, VII. p. 393. 1906.

[2] *Zeitsch. f. physik. Chem.* LVI. p. 105. 1906.

conductivity found, viz., in the experiment quoted, from 882 to 1434 gemmhos or 63 %.

A similar experiment with caseinogen, showing equal increase of conductivity by adding equal amounts of KCl to the same solution before and after the action of trypsin, is given in my previous paper[1].

One more experiment may be quoted in support of the results obtained above:

A 5 % solution of gelatin was taken. The gelatin had been soaked, in the ice-chest, in repeated changes of distilled water, the first three or four changes being slightly acidified with acetic acid, and with the addition of a piece of thymol. The gelatin was then dried on muslin in a current of dry air at 40° and then in a desiccator.

The specific conductivity of this solution was, at 39°, 159 gemmhos; that of the water being 5·75, the conductivity of the gelatin solution was 153·25. The corresponding value of a 5 % solution of the gelatin before washing was 760 gemmhos.

Now it might be supposed, if the increase of conductivity by trypsin were produced by some change facilitating the movement of ions already present, without actually increasing the number of ions present, that the observed increase would be greater the greater the number of ions originally present, naturally within such limits as not to retard the action of the enzyme itself.

To one sample of a 5 % solution of the above-mentioned washed gelatin a small amount of KCl was added, so that its conductivity was raised to 830 gemmhos, *i.e.* slightly higher than that of the gelatin before washing; this rise of 671 gemmhos would correspond to the presence of sufficient KCl to make the solution approximately $\frac{n}{240}$. To this were added 1 c.c. $\frac{n}{2}$ NH_3 and 1 c.c. of 2 % trypsin, and the same amounts to another sample of the washed gelatin solution without addition of KCl. After 24 hours' digestion the conductivities of these two solutions had risen by:

The original solution	... ...	2595 gemmhos
,,	+KCl	2470 ,,

That is, the one with the greater number of original ions showed slightly *less* change. The difference is rather too large (4·8 %) to be an experimental error and may be due to retardation of the action of enzyme by the KCl, though one would not expect any perceptible effect from so small a quantity of electrolyte.

The conclusion to be drawn from the foregoing considerations is that changes of such properties of a solution as those known as "viscosity" or internal friction are insufficient to account for more than a small fraction of the increase in electrical conductivity under trypsin digestion.

[1] *Loc. cit.* p. 15 of reprint.

The fact that, notwithstanding their "viscid" character, solutions of gelatin and other colloids have no greater effect in diminishing the conductivity of electrolytes to which they were added than has the addition of non-colloidal non-conductors, such as glycerin or alcohol, is of a somewhat puzzling nature. It shows, indeed, that a distinction must be drawn between the internal friction of a solution, as measured by its rate of flow through a narrow tube, and that property in virtue of which it causes a diminution of electrical conductivity by retarding the movement of ions present in other solutions to which it may be added. We may, for convenience, distinguish the two properties as "mechanical" and "electrical" viscosity. Arrhenius[1] points out that the influence of non-electrolytes on the conductivity of electrolytes is not in direct relation to their "fluidity," for example: the internal friction of 1 % cane-sugar (by volume) is 1·046 ($H_2O = 1$), 2·2 % methyl-alcohol has the same value, yet the conductivity of KCl solutions is lowered by the cane-sugar by 3 % and by the methyl-alcohol by 3·85 %.

In this connection the following experiment is of interest:

The following substances were taken in 5 % solution by weight; their rate of flow through Ostwald's viscosimeter and the effect of adding the same amount of electrolyte to each were determined as follows:

Temp. 29·9° C.

	Time of flow in minutes	Ratio
Water	44	1·0
Methyl-alcohol	53	1·2
Glycerin	50	1·14
Cane-sugar	48	1·09
Raffinose	51	1·16
Brucin acetate	51	1·16
Gum arabic	165	3·75
Caseinogen in ammonia	210	4·76
Gelatin	960	21·8

The increase in conductivity produced by the addition of 1 c.c. normal KCl to 9 c.c. o each of the above respectively was:

	Increase by KCl	Percentage diminution for 1 % non-electrolyte
Water	13,218	0
Methyl-alcohol	12,289	1·41
Glycerin	12,103	1·73
Cane-sugar	12,356	1·30
Raffinose	13,000	1·02
Brucin acetate	11,575	2·48
Gum arabic	11,126	3·17
Caseinogen in ammonia	10,000	4·85
Gelatin	12,187	2·06

[1] *Loc. cit.* p. 495.

It will be obvious that the two properties in question have no direct relation to one another. This is especially striking in the case of the colloids. For example, the "mechanical viscosity" of gelatin is $\frac{21{\cdot}8}{1{\cdot}14} = 19{\cdot}1$ times that of glycerin, whereas the "electrical viscosity" is only $\frac{2{\cdot}06}{1{\cdot}73} = 1{\cdot}9$ times that of the same body. The same fact comes out on comparing other pairs of values.

The "electrical viscosity" of gelatin in the "gel" state at 15° C. is practically the same as at 29·9°, viz. 2·3.

It may be thought, perhaps, that differences in electrolytic dissociation of the KCl in these several cases may account for the differences in conductivity. In order to test this, determinations were made of the concentration of Cl^- ions in each case. The following concentration battery was used, the content in Cl^- ions being thus obtained indirectly[1]:—

Hg | Calomel | Normal KCl | 3·5*n* KCl | Solution to be tested | Calomel | Hg.

When $\frac{n}{10}$ KCl was taken in the right-hand electrode the potential difference of the battery at 29·9° C. was 0·0632 volt, when $\frac{n}{10}$ KCl in 5 % glycerin was taken it was 0·0633, the higher value of course being due to a diminution of the Cl^- ions present. It is, however, so small as to be practically within the limits of experimental error. The same value, within 0·0001 volt, was obtained for the remaining solutions with the exception of methyl-alcohol and brucin acetate. In the case of the former the potential difference was 0·0580 volt, *i.e.* the concentration of Cl^- ions was *greater* than in the pure KCl; this is obviously due to impurity. The value in the case of brucin acetate was 0·0715 volt, showing a considerable lowering of the concentration of Cl^- ions, probably due to secondary chemical changes; the low value of the conductivity is therefore not to be taken as dependent solely on the "electrical viscosity" of the solution. It is also necessary to refer to caseinogen; since alkali is inadmissible in a calomel electrode, the caseinogen solution was neutralized with acetic acid, so that the greater part of the caseinogen was thrown down, a fact making the value of the potential difference not that of an actual 5 % solution.

Brucin acetate and raffinose were chosen in the above experiment as being crystalloids with large molecules. The molecular weight of

[1] See Ostwald-Luther, *Physik Chem. Messungen*, 1902, pp. 373 sqq.

the former being about 450 and that of the latter 504. The low values of their internal friction justify our regarding them as crystalloids, although they show the Tyndall-phenomenon of reflecting polarized light at right angles when a beam of bright light is passed through them, a fact indicating the presence of ultra-microscopic particles usually regarded as characteristic of colloidal solutions[1].

I would venture to suggest, with considerable hesitation, that the key to this want of relationship between the "mechanical" and "electrical" viscosities in the case of colloidal solutions may perhaps be found in the nature of these solutions as suspensions of ultra-microscopic particles in a very dilute true solution (Hardy). In such cases as that of caseinogen a greater proportion of the body may be in true solution than in the case of gelatin. There may be some difference in the degree of obstruction to the movement of ions according as these collide with solid particles of a size greater than that of molecules, or whether fluid friction is the chief retarding factor[2]. To use a rough illustration, a man in walking from one place to another may be less obstructed by having to thread his way through a crowd of people, occasionally coming into collision, than if he were obliged to wade through a pool of treacle.

The electrical charges of the particles in colloidal solutions may also play a part in diminishing the number of collisions, a negatively charged ion in passing between a number of similarly charged particles would, presumably, owing to mutual repulsion, not be subject to as great a number of collisions as if passing between particles carrying charges of opposite sign to that of its own charge. The matter is, however, of much complexity, although probably capable of experimental investigation[3].

Increase of Inorganic Ions.

The results of the preceding section have shown that the facilitation of the movement of ions already present is insufficient to account for more than a small fraction of the actual increase in conductivity during trypsin digestion. It follows, therefore, that there must be a production of new electrolytes.

[1] See Lobry de Bruyn et Wolff, *Rec. Trav. Chim. des Pays Bas*, xxiii. p. 155. 1904.

[2] See Carse and Laby, *Proc. Camb. Phil. Soc.* xiii. p. 287. 1906.

[3] See the discussion by Hardy, This *Journal*, xxxiii. p. 281. 1905. The results obtained by me are in close agreement with those of Hardy. The work of Bousfield on "Ionic size in relation to conductivity," *Phil. Trans.* A. 206, p. 101. 1906 is of interest also in this connection.

Since inorganic salts are, as a rule, better conductors than organic electrolytes, it is well to turn our attention in the first place to the possibility of a production or setting free (in the sense of ionization) of inorganic electrolytes.

Although there is considerable evidence that inorganic salts are present in an adsorbed form in the majority of protein bodies[1], their percentage amount is not great. At the same time, owing to their superiority over organic electrolytes as conductors, it is necessary to take into account the possibility of their becoming ionized in the process of tryptic digestion. It appears indeed probable that, the products of such action being less colloidal than the original substrate, their adsorbing power for electrolytes would not be so great. As will be seen, however, I have found it a matter of considerable difficulty to obtain direct evidence of the presence of a larger percentage of inorganic ions in the product of a trypsin digestion than before the action of the enzyme. At the best, moreover, the increase of conductivity could be only partially explained in this way. A particular sample of gelatin contained 0·0912 % of ash. This gelatin in 8·5 % solution gave an increase of conductivity after 46 hours' action of trypsin of 1776 gemmhos. If the whole of the ash had consisted of as good a conductor as KCl a percentage content of the gelatin therein of 1·3 % would have been necessary to produce this increase.

The first experiment I performed was to determine by Neumann's method the calcium content of the filtrates after precipitation by tannin of gelatin solutions before and after the action of trypsin. The values obtained were—for 9 grms. of gelatin:

Undigested	...	...	...	0·1043 grm.
Digested	...	...	...	0·1096 grm.

The difference in favour of the latter is too small to be of much value as evidence. The actual values given do not represent the absolute calcium contents of the gelatin in question, since I found that the nitric acid used for oxidation contained a small amount of calcium. The same quantity of acid being used makes the relative values correct.

The explanation of this negative result is I think to be found in the observation of Cramer and Swale Vincent[2] that the inorganic constituents of various organic bodies are set free when these substances

[1] See a paper by the present author, *Biochem. Journ.* I. p. 175. 1906.

[2] This *Journal*, xxx. p. 150. 1903.

are acted upon by various precipitants of basic bodies. I find that this is the case when gelatin is precipitated by tannin[1]. So that all the calcium would be found in the filtrate in both cases.

This method being found of no value for the purpose I tried two different ones. In the first place, on the assumption that chlorine would be one of the components of the ash constituents, I estimated the change of concentration of Cl^- ions by a concentration battery of the kind already described:

100 grms. of 15 % gelatin + 1 c.c. $\frac{n}{1}$ NaOH + 1 grm. trypsin at 38° for eight days. Neutralized by addition of 1 c.c. $\frac{n}{1}$ H_2SO_4. Placed in one calomel electrode, the other containing $\frac{n}{1}$ KCl. The E.M.F. found was 0·1363 volt.

Calculating by Nurnst's formula the concentration (as KCl) of the chlorides of the gelatin, assuming complete dissociation in the digest and 0·68 dissociation of the normal KCl, the value comes out to be $\frac{n}{110}$ or 0·068 %.

A similar preparation in which the trypsin had previously been heated to 100° gave E.M.F. 0·1415 or as KCl $\frac{n}{130} = 0{\cdot}058$ %.

In this experiment there was evidence, therefore, of an increase of chlorine ions corresponding to 0·01 % of KCl. But I would not lay much stress on this result, since the solutions were of a complex nature and there is some uncertainty as to the actual process at the electrode.

In the second place I have made some experiments by filtration of digested and undigested gelatin through Martin's gelatin filter[2]. For this purpose I used D'Arsonval's apparatus for sterilization by means of liquefied carbon dioxide. The brass tube, into the lower end of which was screwed the porcelain candle impregnated with gelatin and treated with formalin, was filled with the solution to be filtered and connected at the top to a cylinder containing liquid carbon dioxide. By this means the pressure was kept continuously at about 50 atmospheres until sufficient filtrate had been collected. In the case of the undigested gelatin it was necessary to keep the tube warm by surrounding it with a coil of pipe through which a current of water warmed to about 50° C. was allowed to flow. It was not found that the filtrate contained any considerable amount of dissolved carbon dioxide, not sufficient to come off in bubbles; this was perhaps due to the fact that there was a layer of toluene on the surface of the liquid in the filter which prevented easy

[1] "Adsorption phenomena," *loc. cit.* p. 209.

[2] This *Journal*, xx. p. 364. 1896. See also Reid, This *Journal*, xxvii. p. 161. 1901, and Craw, *Proc. Roy. Soc.* B. lxxvii. p. 311. 1905.

access of the carbon dioxide. In all cases any dissolved CO_2 was driven off by boiling in vacuo before determining the conductivity; the water lost was determined by weighing before and after exposure to vacuum and replaced by the necessary amount of distilled water. The total solids in a measured volume of each filtrate were estimated by evaporating to dryness in a platinum capsule on the water-bath and drying in a toluene oven at 110°. This solid was ashed by carefully heating to low redness by a Bunsen burner until the carbon had only just disappeared. The results were as follows:

	Amount of filtrate in c.c.	Total solids in grms.	Ash in grms.	Percentage	
				Total solids	Ash
Undigested, Exp. 1	75	0·5032	0·0310	0·6755	0·041
Digested ,,	170	3·8	0·0663	2·23	0·0391
Undigested, Exp. 2	108	0·1748	0·0453	0·1618	0·042
Digested ,,	100	2·109	0·0457	2·109	0·0457

In experiment 1 a 3% solution of Nelson's gelatin washed previously with dilute acetic acid and distilled water was taken, and in experiment 2 an 8·5% solution of Coignet's gold label gelatin.

It will be seen that there is no difference in ash of the filtrates from the digested and undigested preparations. At the same time I am inclined to think that the action of the carbonic acid dissolved under pressure was to split off the adsorbed electrolytes from the unaltered gelatin. The conductivity of the original solution was 1854 gemmhos, whereas that of the filtrate was 2881 gemmhos, after boiling off in vacuo dissolved CO_2. These experiments cannot therefore be regarded as decisive.

In the case of gelatin the evidence on the whole seems to indicate that the splitting-off of inorganic electrolytes takes but a small share in the increase of conductivity.

In confirmation of this I may refer to an experiment in which it was found that an equally great rise of conductivity took place in the case of gelatin which had been repeatedly extracted by distilled water as in that which had not been so treated. Also that a preparation of Harnack's "ash-free" albumin, obtained from Grübler, gave as much increase as ordinary egg-white.

Caseinogen is believed to give off its phosphorus under the action of trypsin as inorganic phosphate. In order to obtain evidence as to the share this might have in the increase of conductivity I made, in conjunction with Aders Plimmer, a number of determinations of the

phosphorus split off at various stages of the reaction[1]. We found that only about one-fourth of the total phosphorus in the filtrate after precipitation by tannin is in the form of inorganic phosphate, the remainder being in the form of a peptone-like body. The time-course of the separation of the inorganic phosphate is, in its earlier stages, parallel to the course of the conductivity, but, after some three or four hours, the former ceases to increase whereas the latter continues to rise fairly rapidly for several further hours. A little consideration will also show that the amount of inorganic phosphate produced is too small to account for more than a part of the rise of conductivity. Thus: in experiment No. 4 (p. 443 of the paper referred to) it was found that a 5 % solution of caseinogen increased in conductivity by 3580 gemmhos in 14 days and 0·098 grm. of phosphorus, reckoned as P_2O_5, had been split off per 100 c.c. of solution. Of this one-quarter is in inorganic form and, assuming it to be Na_2HPO_4, would account for a rise of conductivity of 1000 gemmhos or about 27 % of the observed amount. It appears then that, in those cases where trypsin separates off inorganic phosphates, these salts are capable of producing a certain part of the increase in electrical conductivity which takes place. At the same time it will be seen from the data given that there is some additional cause even in the case of caseinogen. The same fact can indeed be shown in another way. In the paper by Aders Plimmer and myself, above referred to, the action of dilute alkali on caseinogen is described. It was found that 1 % caustic soda splits off the whole of the phosphorus from caseinogen in 24 hours, leaving a body free from phosphorus but otherwise very similar in its properties to the original caseinogen. Now this body, which we propose to call "apocasein," shows on digestion by trypsin almost as great a rise of conductivity as caseinogen itself. In one experiment there was a rise of 2730 gemmhos in 24 hours, which must clearly have been due to some process other than the separation of inorganic phosphate.

Production of Peptones.

Recent measurements by Walter Neumann[2] have shown that peptone as produced by the action of pepsin on fibrin and prepared by Siegfried's method[3] is a body of strongly acid properties and possessed of considerable electrical conductivity. The molecular conductivity of

[1] This *Journal*, xxxiii. p. 439. 1906.

[2] *Zeitsch. f. physiol. Chem.* xlv. p. 216. 1905.

[3] Curt Borkel, *Zeitsch. f. physiol. Chem.* xxxv. p. 289. 1902.

"pepsin-fibrin-peptone α" is from 15 to 20 recip. ohms according to concentration of solution and on the basis of a molecular weight of 515. This seems to be a very high value, and naturally suggests the presence of inorganic salts; at the same time great care was given to the exclusion of this source of error.

If, then, the bodies called "peptones" have so considerable an electrical conductivity it would seem that they are responsible for the greater part of the rise in trypsin digestion.

In support of this view the following results may be brought forward:

Tannin in acid solution does not precipitate peptone, or only certain of the bodies known by this name[1]. It was therefore of interest to compare the curve of the nitrogen content of the filtrate from tannin precipitation with the curve of the conductivity in trypsin digestion. This has been done and the results are given in the curves of Fig. 2. The values are taken from two separate digestions of caseinogen and, for comparison, some other results are included as follows:

Curve A represents the time-course of the nitrogen contained in the filtrates from precipitation by hot trichloracetic acid, *i.e.* the difference between its ordinates and the upper boundary of the figure represents the amount of unaltered caseinogen present. It will be noted that all through after the second hour there was a very slight precipitate with trichloracetic acid, but that this was constant and evidently due to a body not attacked by trypsin, possibly nuclein.

Curve B represents the nitrogen in the filtrate after $\frac{1}{2}$ saturation with $ZnSO_4$; *i.e.*, regarded in the same way as A, it represents the proteose present at each stage.

Curve C in the same way represents the nitrogen in the tannin filtrate.

Curve D the nitrogen of the phospho-tungstic filtrate. The precipitation was performed in dilute solution and the curve therefore represents, in the main, the mono-amino-acids produced.

The numerical values of ordinates given on the left-hand side refer to the nitrogen curves and are decigrams of nitrogen per cent.

Curve E, in dotted lines, is that of the electrical conductivity. The origin is taken from the level of *O* in the scale of the right-hand side of the figure, which gives conductivity values in gemmhos. This was done in order not to confuse the curve with that of the tannin filtrate.

[1] See P. Mey, *Zeitsch. f. physiol. Chem.* XLVIII. p. 81. 1906.

It will be seen that the curve of the conductivity follows closely that of the nitrogen in the tannin filtrate; it therefore serves as a good indicator of the amount of peptone and amino-acids present in the digest at any stage of the reaction.

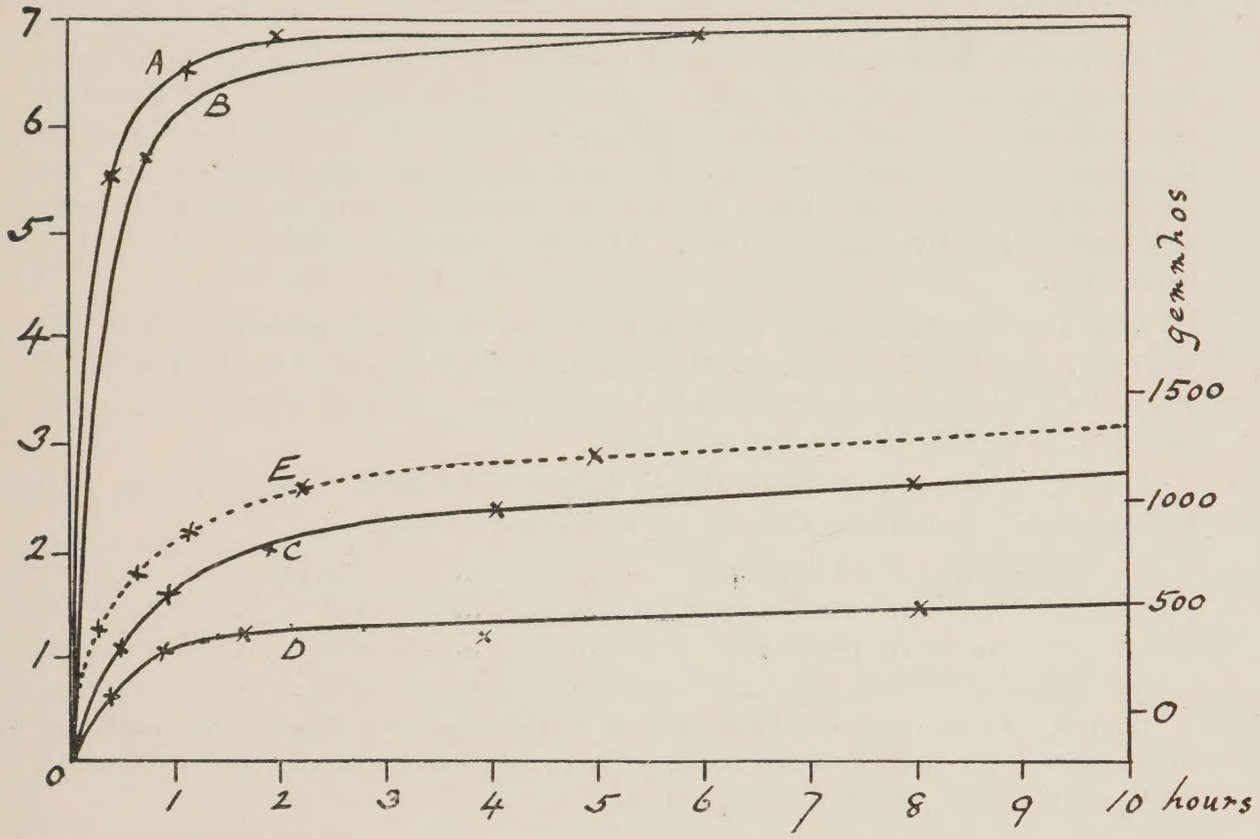

Fig. 2.

In the case of gelatin it is to be presumed that the chief electrolytes produced are the gelatin-peptones, since it is doubtful whether amino-acids are formed in any considerable degree. That organic electrolytes are produced is shown also by the results of the gelatin filter experiments already described; the filtrates from the digested and undigested samples although containing, within the limits of experimental error, the same percentage of inorganic salts, differed considerably in electrical conductivity:

Undigested	...	...	2470 gemmhos.
Digested	...	...	3615 gemmhos.

The conductivity of the digested gelatin before filtration was 3630 gemmhos, so that practically all the electrolytes were such as to pass through gelatin.

In confirmation of the results of Walter Neumann, referred to above, I have found the electrical conductivity of various peptone

preparations which I have been able to obtain to be very considerable. At the same time it must be stated that none of my preparations were ash-free, so that the values of the electrical conductivity obtained had to be diminished by that of the inorganic constituents, and cannot therefore be regarded as of any great accuracy.

The value of the conductivity of the ash constituents was determined by evaporating to dryness a weighed quantity of the peptone solution on the water-bath. By this means the concentration of the solution used was obtained. The dry residue was next heated just sufficiently to char the organic material. The char was extracted with distilled water. The residue was then completely ashed at a low red heat, dissolved in the first extract and made up to original volume of solution. As a rule a very small amount of ash remained undissolved.

Prep. 1. "Ampho-peptone" prepared by myself, by Kühne's method. Faintly acid to litmus. Insoluble in alcohol. 4·9 % solution. Conductivity at 39·6° = 1250 gemmhos. That of the ash was 403 gemmhos, so that the peptone itself had a conductivity of 847 gemmhos, or for 5 % solution, 864 gemmhos.

Prep. 2. A preparation obtained from Grübler and labelled "Ampho-peptone nach Kühne puriss." Contained no barium nor ammonium sulphate.

	Conductivity of 3·7 % solution	...	...	555 gemmhos
	,, ,, ash	...	...	120 ,,
∴	,, ,, peptone	...	...	435 ,,
or	for 5 % solution	...	...	588 ,,

Prep. 3. An alcoholic (96 %) extract of Witte's peptone. The peptone contained water, since it was not previously dried. The extract was evaporated to dryness and a 7·6 % solution made, which was acid to litmus.

	Conductivity of solution	...	...	20,150 gemmhos
	,, ,, ash	...	...	11,100 ,,
∴	,, ,, peptone	...	...	9,050 ,,
or	for 5 % solution	...	...	5,940 ,,

Another determination of the same preparation in stronger solution gave 4970 gemmhos reckoned for 5 % solution. It appears from the above result that some substance soluble in alcohol of about 80 % of high conductivity is present in Witte's peptone.

When a "peptone" preparation of Hopkins and Williams was dried in a toluene bath it was found that 96 % alcohol extracted only 0·02 grm. from 25 grms. of the peptone, *i.e.* less than 0·1 %. This was found to consist almost entirely of inorganic salts. At the same time a small amount of organic substance was present which appeared to have a high conductivity. In this particular preparation then there is only a trace of a body soluble in 96 % alcohol. The various commerical "peptones" are known to have very variable compositions, so that the result given above is not be taken as contradicting those of Pick.

On the whole it is clear that the organic constituents of peptone preparations have a high conductivity, although, as previously stated, the considerable percentage of ash makes the exact values somewhat uncertain.

An attempt was made to obtain a purer preparation by precipitation with phospho-tungstic acid, thoroughly washing the precipitate with dilute sulphuric acid and decomposing with barium hydroxide. It was found that 1·71 grm. of the body obtained contained 0·156 grm. of ash (*i.e.* 9·1 $^0/_0$) which almost entirely dissolved with effervescence in hydrochloric acid, a small quantity being insoluble. The ash therefore presumably consisted of barium carbonate with a little sulphate, and the preparation was a barium "peptonate." Peptone appears to be a sufficiently strong acid to displace carbonic acid from its combination with barium.

A 15 $^0/_0$ solution of the substance had a specific conductivity of 5480 gemmhos.

In the filtrate from tannin precipitation of a trypsin digest there are also present various mono-amino-acids to which attention will now be given. In the case of gelatin, amino-acids are only split off by trypsin comparatively slowly, so that the rise of conductivity here must be mainly due to the "gelatin-peptones" formed.

Production of Amino-acids.

As shown by the curve D on Fig. 2 there is from the beginning a certain production of bodies not precipitable by phospho-tungstic acid. This result is in agreement with those of previous observers. Tyrosin, in fact, is usually to be seen deposited in less than 24 hours when caseinogen is used as substrate.

Although scattered determinations of the conductivity of some amino-acids are to be found in various papers, I have been unable to find any relating to leucine, which is that one of most interest in the present connection, being the most abundant of those produced in the tryptic digestion of the proteins dealt with here.

I have also thought it worth while to make determinations of the conductivity of some other amino-acids under the same conditions of temperature, etc., as the digestion experiments were made[1].

Some preliminary experiments showed that the conductivity of the mono-carboxylic series, glycocol, leucin, etc. was by no means high. It was therefore of great importance to obtain preparations free from inorganic salts. As slight contamination with nearly related amino-acids was of much less consequence, it seemed that the fractional distillation of the ethyl-esters by Emil Fischer's method was the best available. The esters of the mono-carboxylic series can be hydrolyzed by boiling with water and by this means pure solutions of the amino-acids can be obtained.

[1] For the theory of amphoteric electrolytes see the papers by James Walker in *Proc. Roy. Soc.* LXXIII. p. 156. 1904, and LXXIV. p. 271. 1904.

Owing to the considerable time taken in these manipulations I have only been able to prepare glycocol, alanine, leucine and glutamic acid by the ester method.

Glycocol. The ethyl-ester was prepared from the hydrochloride as supplied by Kahlbaum. This was distilled under diminished pressure, a considerable part remaining in the flask. The distillate was hydrolyzed by boiling for several hours with conductivity water in a Jena glass flask with a reflux condenser also of Jena glass. The free amino-acid was recrystallized from dilute alcohol, washed with alcohol and ether and dried. A $\frac{m}{10}$ solution of this was found to have a conductivity of 10·4 gemmhos; the water used had a conductivity of 1·9 gemmhos, so that the value for the glycine itself was 8·5 gemmhos.

The glycocol used had a melting-point of about 240°, determined by the method of Kutscher and Otori[1], but it began to char before melting.

That the setting-free of glycocol produces an increase in conductivity is shown by the experiments of Euler[2], who found such to take place in the action of erepsin on glycyl-glycine. I have found that a similar rise takes place in the action of trypsin on glycyl-tyrosin, in fact a 5 % solution, which was not free from ammonium chloride, showed a rise of about 900 gemmhos in four days. The "biuret-base" of Curtius, which is tri-glycyl-glycine-ethyl-ester[3], also shows an increase in conductivity on hydrolysis. Further experiments on polypeptides are in progress. For the present purpose it is sufficient to note that the setting-free of glycine produces a rise in conductivity.

Leucine was obtained by fractional distillation under reduced pressure of the ethyl-esters of the first crop of crystals coming out on concentration of a tryptic digestion of caseinogen, the tyrosin having first been filtered off. A small amount of distillate was collected below 40° at a pressure of 35 mm. Hg, this consisted chiefly of alcohol and was rejected. Nothing more came over until a bath-temperature of 75°—80° was reached, at which temperature and at a pressure of 2 mm. Hg a considerable quantity was collected. Again, no further distillate came over until 120° in oil-bath was reached, when a second large amount was collected.

The fraction coming over at 80° was no doubt nearly pure leucine-ester. It was hydrolyzed by boiling with water as described for glycine, most of the leucine crystallizing out on cooling.

The melting-point was found to be, in sealed tube, 280°. The value found by Fischer was some 7° or 8° higher. Cohn found 275°—276°. The differences are probably to be explained by the effect of varying rates of heating.

The solubility of the preparation in water at 38° was 3·17 %.

Its rotatory power was, in 21·9 % HCl,

$$[\alpha]_D^{16^\circ} = +18{\cdot}16^\circ.$$

[1] *Zeitsch. f. physiol. Chem.* XLII. p. 193. 1904.

[2] *Zeitsch. f. physiol. Chem.* LI. p. 213. 1907.

[3] Curtius, *Ber. d. D. Chem. Ges.* XXXVII. p. 1284. 1904.

In deci-molecular solution the electrical conductivity was very low, only 9·23 gemmhos at 39·4°. When one considers that a fairly good distilled water has, at 18°, a conductivity of 2—3 gemmhos, $\frac{n}{10}$ KCl 11,190 gemmhos, the very slight ionization of leucine in a solution of the above strength is obvious.

When the conductivity of a $\frac{m}{10}$ solution is calculated from that given by a 3·17 % the value of 6·4 gemmhos was obtained, and from a 12·5 % solution (leucine less pure and more soluble) only 3·6 gemmhos.

From the values of the physical constants given the preparation may be taken as practically pure leucine. It possibly contained a small amount of amino-valerianic acid, but this would affect the conductivity only to a very slight degree.

The sharp fractionation of the esters of the first crop of crystals is interesting and indicates that this crop consisted almost entirely of leucine and glutamic acid. It will be remembered that it was shown by Fischer and Abderhalden[1] that all the proline and phenyl-alanine contained in caseinogen is to be found, after the action of trypsin, in a complex polypeptide which contains in addition other amino-acids. In the case before us this polypeptide would be left in the mother-liquor, which would also probably contain the more soluble amino-acids set free by trypsin, such as glycine and alanine. It would appear therefore that the simple composition of the first crop of crystals is to be accounted for in this way. The small fraction of aspartic acid produced would probably be partly contained in this crop, but it was not specially tested for.

Alanine. A preparation obtained from Merck was recrystallized five times from dilute alcohol, but it was found that by this means it could not be obtained free from ash. The ethyl-ester was therefore made, distilled and hydrolyzed as in the cases of glycine and leucine; the ester distilled over under a pressure of 9 mm. Hg at 47° temperature of water-bath.

The body so obtained had a melting-point of 279° and a conductivity in $\frac{m}{10}$ solution (0·891 %) of 10·0 gemmhos.

It will be noted that this value is slightly higher than that of either glycine or leucine, and, although at first sight anomalous, it is in agreement with the determinations of Winkelblech[2], who found that alanine is at the same time both a stronger acid and a stronger base than either of the two former.

Phenyl-Alanine. Although not strictly belonging to the amino-acids produced in tryptic digestion it seemed of interest to compare the conductivity of phenyl-alanine with alanine.

[1] *Zeitsch. f. physiol. Chem.* xxxix. p. 81. 1903.

[2] *Zeitsch. f. physik. Chem.* xxxvi. p. 587. 1901.

Accordingly I prepared a specimen of synthetic phenyl-alanine by the method of Emil Fischer[1]. This had a melting-point of 250° and was soluble in water at 38·25° to the extent of 2·21 %. A solution of this strength had a conductivity of 35 gemmhos. A $\frac{m}{10}$ solution being 1·652 % its conductivity calculated from the above would be 26·2 gemmhos.

The effect of the phenyl group is thus seen to be to raise the conductivity as compared with alanine.

Proline. Dr Aders Plimmer kindly placed at my disposal a specimen prepared by him by the acid hydrolysis of gelatin. This had a melting-point of 206°—207°. In solution of 1·177 % it had a conductivity of 13·1 gemmhos. Calculated for $\frac{m}{10}$ solution (=1·1513 %) the value is 12·8 gemmhos.

Proline is of course not strictly an amino-acid, having a NH group and not NH_2. Since the conductivity of an amphoteric electrolyte is, so to speak, a compromise between its properties as a base and an acid, the acid function predominating, it would be expected that the conductivity of proline would be low, since the NH has stronger basic properties than NH_2 and therefore opposes the effect of the carboxyl to a more considerable degree than does the latter.

In all probability the value for the conductivity of proline given above is rather too high, since the sample used was not specially purified.

From the above results as a whole it appears that the mono-carboxylic mono-amino acids contribute comparatively little to the rise of conductivity in tryptic digestion.

Aspartic acid. In the case of the esters of the di-carboxylic amino-acids mere boiling with water does not smoothly hydrolyze them to the corresponding acids, so that the method used above does not present any great advantage over that of repeated recrystallization from water, since the preparation obtained by boiling with barium hydroxide would have to be purified in this way.

I took therefore a specimen of aspartic acid obtained from Hopkins and Williams and recrystallized it from conductivity water until the mother-liquors of two consecutive recrystallizations showed the same conductivity. For this purpose it was necessary to repeat the process five times. The acid thus obtained was only soluble to the extent of 0·853 % in water at 38°. The conductivity of this solution at 32·6° was 912 gemmhos. Calculated from this value that of a deci-molecular solution (=1·331 %) would be 1420 gemmhos. The value calculated from that of a 0·3035 % solution amounts to 2520 gemmhos. The acid used in these determinations had a specific rotatory power of

$$[\alpha]_D^{16^\circ} = 25{\cdot}15^\circ \text{ in } 21{\cdot}9\ \%\ \text{HCl}.$$

[1] *Ber. d. D. chem. Ges.* XXXVII. p. 3062. 1904.

Aspartic acid then, contrary to the mono-carboxylic acids, has a high conductivity. This is in agreement with its other properties as a fairly strong acid[1]. In fact when we consider that one carboxyl group is more than sufficient to neutralize the effect of the NH_2 we see that the second COOH is more or less free to exert its H^+ ion-producing power.

Glutamic acid. This body is of much importance in the hydrolytic action of trypsin, since it is contained in considerable proportion in most proteins[2].

I have had some difficulty in preparing it in a state of sufficient purity for conductivity determinations, but found the best method to be from the hydrochloride which separates out from the products of hydrolysis of casein on saturation with HCl[3]. This was recrystallized and then decomposed by moist silver oxide. To free the solution from ammonia it was evaporated in vacuo at 40° and the silver glutamate decomposed in the usual way by sulphuretted hydrogen. The free acid was then recrystallized five times from conductivity water.

It was soluble to the extent of 1·76 % in water at 38·5°.

Its melting-point was 195° by the method of Kutscher and Otori. In my hands, however, this method gives results about 5° lower than the ordinary method of immersion in sulphuric acid.

Its specific rotatory power was

$$[\alpha]_D^{17^\circ} = +29{\cdot}6^\circ$$

when dissolved in equivalent amount of HCl. This value is very slightly below that found by Fischer; my preparation was probably racemized to a small extent by the silver oxide used.

The conductivity of a 1·76 % solution was, at 39·4°, 857 gemmhos. From this value that of a $\frac{m}{10}$ solution (1·471 %) would be 716 gemmhos.

A second determination was made on a specimen kindly placed at my disposal by Dr Aders Plimmer. This was obtained from hydrolysis of gelatin. It had a melting-point of 205° and was soluble in water to the extent of 1·975 % at 39°. The conductivity of this solution was 1104 gemmhos or for $\frac{m}{10}$ 822 gemmhos. It was therefore not quite so pure as the first preparation, but had only been once recrystallized.

A third preparation was made from the di-ethyl-ester. The recrystallized glutamic acid hydrochloride was esterified by HCl gas in the usual way[4]. The ester, set free by potassium carbonate, distilled over at 1·5 mm. Hg and temperature of oil-bath of 115°—120° C. It was hydrolyzed after removal of the phenyl-alanine ester by ether[5], by heating on the water-bath with a known amount of barium hydroxide, which was afterwards removed

[1] See Walden, *Zeitsch. f. physik. Chem.* VIII. p. 482. 1891.

[2] See the table in Cohnheim's *Chemie d. Eiweisskörper*, 2te Aufl. 1904, pp. 42—47.

[3] For two samples of this substance my thanks are due to Mr P. Hamill of the Cambridge Physiological Laboratory.

In the filtration of small amounts of the hydrochloride from strong hydrochloric acid the platinum-sieve filter will be found of much service. (Aders Plimmer and Bayliss, This *Journal*, XXXIII. p. 439. 1906.)

[4] Fischer, *Ber. d. D. chem. Gesellsch.* XXXIV. p. 433. 1901.

[5] Fischer, *Unters. über Amino-säuren, Polypeptiden u.s.w.* S. 59. 1906.

by the addition of the equivalent quantity of sulphuric acid. The glutamic acid was five times recrystallized from conductivity water and had then in $\frac{m}{20}$ solution at 39·3° C. a specific conductivity of 498 gemmhos. Its melting-point was 203° C., it was soluble in water at 37·2° C. to the extent of 1·92 %, and this solution had a conductivity of 1046 gemmhos at 39·3°.

Although the conductivity of glutamic acid is, then, not so high as that of aspartic acid, it is, nevertheless, very considerable.

Since glutamic acid evidently plays a considerable part in the rise of conductivity under the action of trypsin it is to be expected that bodies like gliadin, which contains more than 37 % of glutamic acid[1], would give an unusually high increase in these circumstances.

The substance used was obtained by the extraction of commercial gluten with 70 % alcohol at 39°. A 2·5 % solution of this in dilute ammonia showed an increase of 2600 gemmhos in 24 hours or double that shown by caseinogen.

When we consider that comparatively strong acids like aspartic and glutamic are produced by the action of trypsin it is not surprising to find that under some conditions a trypsin digest may actually become acid to litmus. I noticed this in a digest of caseinogen made for the purpose of obtaining tyrosin and in which 2 c.c. of ·880 ammonia per litre of 10 % caseinogen were added at the commencement. Dr Hopkins informs me that he also has made the same observation.

This circumstance is of considerable importance in two respects, one practical and the other theoretical. It shows that, if a trypsin digest is required to proceed as far as possible, alkali must be added during the course of digestion, since trypsin is enormously increased in activity by OH^- ions. Secondly, it is very usual in reactions catalyzed by enzymes that the reaction-velocity falls off considerably below that given by the logarithmic law of mass-action. This was shown by Tammann for emulsin and by myself for trypsin as well as for other enzymes by other workers. Now it was found by Visser[2] that, if the reversibility of the reaction is taken into account, a part of the deviation can be explained, but not the whole; he, therefore, introduced the conception of a change in the intensity of activity of the enzyme during the progress of the reaction, independent of any destruction of the enzyme. In the form of "auto-catalysis" a similar phenomenon is well known in chemical reactions, a typical instance being that of the action of nitric

[1] Osborne and Harris, *Amer. Journ. of Physiol.* XIII. p. 35. 1905.

[2] *Zeitsch. f. physik. Chem.* LII. p. 257. 1905. See also the discussion by Moore in *Recent Advances in Physiology*, 1906.

acid on copper as accelerated by the nitrous acid produced. Such autocatalysis may be positive or negative and I think there can be no doubt that the development of acids in trypsin digestion is an instance of the latter. I hope to return to this point in a future paper.

Lysine. This is the only representative of the di-amino-acids or hexone-bases that I have been able as yet to obtain in a sufficient degree of purity for conductivity measurements. An amount of inorganic impurity inappreciable by chemical means vitiates seriously the accuracy of such determinations.

The picrate was first made by the method of Kossel and Kutscher[1] from the products of acid hydrolysis of caseinogen. This was recrystallized, decomposed by H_2SO_4 and the picric acid removed by extraction with ether. The sulphate was again precipitated by phospho-tungstic acid, washed repeatedly with 5 % pure sulphuric acid, decomposed by barium hydroxide, excess of the latter removed by CO_2, evaporated to dryness on water-bath, extracted with water and the conductivity of the solution of lysine carbonate determined as follows :

Concentration of the solution	...	...	7·4 %
Conductivity at 39·7°	..	...	4500 gemmhos

From this must be deducted the value of barium carbonate dissolved, which was found in a control experiment (without lysine) to be 105 gemmhos. The value is then reduced to 4385 gemmhos.

In order to obtain the free base I redissolved the lysine carbonate, which crystallized out on concentrating the above solution, in a known volume of $\frac{n}{1{\cdot}5}$ H_2SO_4, boiled in vacuo to expel CO_2, and an exactly equivalent amount of barium hydroxide added. Filtered in the open air (in my garden at Hampstead) directly into conductivity vessel.

The concentration of the solution was 1·505 %.

Conductivity at 39·4°, 962 gemmhos.

The conductivity of the ash determined by the method of "charring" was 45 gemmhos, so that the above value must be reduced to 917 gemmhos.

On the basis of this number the conductivity of a deci-molecular solution (=1·46 %) would be 890 gemmhos.

It appears then that the di-amino-acids have a high conductivity, but I do not wish to lay much stress on the exact value given. At the same time the amount of inorganic impurity seems to have been infinitesimal if the conductivity value of the ash be correct. Moreover on complete incineration a just visible film was left on the platinum, too minute to be weighed.

In respect to the hexone bases, it is of interest that scombrine sulphate and clupeine carbonate, which I prepared by Kossel's method, although not acted on by trypsin with any great rapidity, ultimately showed a considerable rise in conductivity.

The scombrine sulphate was not completely free from histone and nucleic acid. A 4·9 % solution increased in conductivity by 4440 gemmhos in 4 days. The ash, however, was considerable and accounted for 856 gemmhos.

[1] *Zeitsch. f. physiol. Chem.* xxxi. p. 165. 1900.

The clupeine carbonate was carefully freed from the above contaminations and gave in 7·3 % solution an increase of 940 gemmhos in 4 days.

Taking into consideration all the facts given in this section it will be obvious that, although the mono-carboxylic mono-amino acids contribute but little to the rise of conductivity under trypsin, the di-carboxylic amino-acids (aspartic and glutamic) play a considerable part, as do also, in all probability, the hexone bases.

This being so, it would naturally be expected that the products of a peptic digestion, even a protracted one, would manifest an increased conductivity under tryptic digestion. Experiment shows that such is the case.

Exp. Caseinogen was digested by pepsin and $\frac{n}{10}$ sulphuric acid for a week. The digest was neutralized by ammonia and evaporated to dryness. The residue, which contained some ammonium sulphate, was made into a 5 % solution in $\frac{n}{2}$ ammonia and subjected to the action of trypsin at 39·7° C. In the course of five days the conductivity increased by 2900 gemmhos.

A word may here be said as to gelatin. In the digest filtered through Martin's gelatin filter obtained in the second of the two experiments described in the third section of this paper, a total nitrogen percentage of 0·628 was found by Kjeldahl's method. Of this 0·123 % was precipitated by tannin and 0·476 by phospho-tungstic acid, so that the filtrate of the digest contained 0·152 % of nitrogen in a form unprecipitated by phospho-tungstic acid, *i.e.* 30 % of the whole. No doubt part of this was split off from the trypsin by auto-digestion, but even if we allow for the whole of the nitrogen contained in the trypsin to have become non-precipitable by phospho-tungstic acid, there remains 22 % to come from the gelatin. This result is in agreement with those of Levene[1], who found amino-acids produced in the digestion of gelatin.

Some properties of Amino-acids.

It remains to refer to certain properties of amino-acids which are of importance in connection with the present subject.

1. *Solubility of mixtures of amino-acids.* It is well known how very much more soluble amino-acids are in solutions of other amino-acids than in water, so that as they are purified their solubility diminishes. This was particularly striking in the case of the glutamic acid described above. Although of course a certain amount was lost in

[1] *Zeitsch. f. physiol. Chem.* xli. p. 8. 1906.

the mother-liquor of each recrystallization it was found that each time more water was required to dissolve the crystals than had been required to dissolve the larger quantity of less pure crystals of the previous recrystallization. It seemed worth while to make some exact measurements of the solubility of these acids in each other.

A particular sample of leucine was soluble in water at 16° to the extent of 12·5 %, in a 5 % solution of glycocol it dissolved to the extent of 16·35 %. With aspartic acid the effect was more marked. In water at 16° aspartic acid was soluble only to 0·324 %, 12·5 % leucine dissolved 10·6 %, or 30 times as much as water.

Since aspartic is a moderately strong acid the idea suggests itself that perhaps a kind of salt, say leucine aspartate, may be formed.

To test this, a mixture of two mols. of leucine and one mol. of aspartic acid in solution was taken and its various properties tested as follows:

The optical activity of the mixture was practically that of a simple mixture, viz., $[\alpha]_D^{16°}$, calculated 20·38°, found 19·93°. When concentrated by evaporation the first crop of crystals was practically pure leucine, having a $[\alpha]_D^{16°}$ of 18·4°, that of the leucine employed being 18·16°. Its electrical conductivity was slightly higher than that of leucine, so that some aspartic acid was contained in it. The second and third crops were not investigated, the fourth crop was mainly aspartic acid, having an optical activity of $[\alpha]_D^{16°} = 20·65°$, that of the aspartic acid being 25·15°. It contained leucine as shown also by its conductivity being less than that of aspartic acid and also its solubility in water was much greater than that of this latter. When the solution of the two acids was allowed to evaporate slowly at room temperature, two distinct sets of crystals formed, one of long needle-shaped crystals near the top of the solution, consisting of leucine and the other small, hard grains at the bottom of the beaker, apparently aspartic acid.

There is, then, no evidence of a salt formation, but it is remarkable that, although leucine is so much more soluble than aspartic acid, yet on concentration the former comes out first, a familiar fact in the treatment of trypsin digests, where the first crop of crystals, although containing glutamic acid, is chiefly leucine.

The question of the electrical conductivity of the mixture will be best discussed in the next section.

2. *The electrical conductivity of certain salts of amino-acids.*

It is well known that when solutions of a strong base and a strong acid are mixed in equivalent proportions, so that a neutral salt is produced, the electrical conductivity of the salt is considerably less than the sum of the conductivities of equi-molecular solutions of the acid and base from which it was formed. A similar phenomenon is seen when one of the reacting bodies is a weak acid or base and the other a strong base or acid. The facts are readily accounted for on the electrolytic and hydrolytic dissociation hypotheses. On the other hand

when both the acid and base are weak the electrical conductivity of the solution of the salt is considerably higher than the sum of its constituents. This fact, which was, I believe, discovered by James Walker in the case of aniline acetate and mentioned by him to Arrhenius[1] has not yet received a complete explanation. It is obvious that, even if the salt were totally dissociated hydrolytically, its conductivity could not exceed the sum of that of its constituent acid and base. There is, in fact, a relatively very large *electrolytic* dissociation of the salt into its ions; but why this takes place is not known. It has been suggested that the phenomenon is due to a peculiar kind of dissociation of ammonium derivatives. To compare with the case of amino-acids to be given presently I give here the results of an experiment with aniline acetate:

A $\frac{m}{20}$ solution of acetic acid had at 21·8° C. a specific conductivity of 330 gemmhos, and a solution of aniline of the same concentration that of 13 gemmhos, the sum being 343 gemmhos. A mixture of equal volumes of $\frac{m}{10}$ solutions of these bodies, so that the salt formed was in $\frac{m}{20}$ concentration, had a specific conductivity of 1518 gemmhos.

If amino-acids formed salts with one another, the one acting as base and the other as acid, it would be expected that the mixture would have a higher conductivity than the sum of its constituents. Before testing this it seemed advisable to make a few determinations of the behaviour of amino-acids when combined with other acids and bases, not amphoteric in nature.

In all the following observations the solutions were mixed in equal volumes of $\frac{m}{10}$ solutions, so that the conductivities given are those of $\frac{m}{20}$ solutions of the salts and the sums are those of $\frac{m}{20}$ solutions of the acid and base in each case. The temperature of the thermostat was 21·8.

Constituents	Conductivity in gemmhos: Sum	Conductivity in gemmhos: Mixture
Leucine and NaOH	9698	2953
Leucine and NH_4OH	235	1548
Leucine and Leucine-ester	82·5	359
Leucine and HCl	17,948	7585
Leucine and acetic acid	333	267

The above results show that leucine behaves normally as a weak acid, both in combination with strong and weak bases. But as a weak base it appears to combine only with strong acids. If it combined with

[1] See Arrhenius, *Zeitsch. f. physik. Chem.* v. p. 18. 1890.

a weak acid, like acetic, the conductivity of the mixture should be greater than the sum of its components, whereas it is less. This latter fact may admit of two different interpretations. Either no combination takes place at all, in which case the fall of conductivity is due to the two acids, acetic and leucine, not being in isohydric concentration, or leucine is such a weak base that acetic behaves towards it as a strong acid. Another possible explanation of the failure to combine is that leucine in solution is in a state of internal anhydride and that, while strong acids can hydrolyze this body and so set free the NH_2 to combine with, weak acids are unable to do so.

Now when leucine is mixed with aspartic acid or glycine the result is similar to that of mixing it with acetic acid, viz. a fall of conductivity as compared with the sum of the components. Thus :

Constituents	Conductivity in gemmhos	
	Sum	Mixture
$\frac{m}{10}$ leucine + $\frac{m}{20}$ aspartic acid, equal parts	443	413
$\frac{m}{10}$ leucine + $\frac{m}{10}$ glycine, equal parts	6·74	3·74

The assumption that the fall of conductivity in the mixture is due to the two solutions mixed not being isohydric is confirmed by the following results on the corresponding fatty acids :—

Constituents	Conductivity in gemmhos	
	Sum	Mixture
$\frac{m}{20}$ isocaproic acid + $\frac{m}{40}$ succinic acid, equal parts	648	482
$\frac{m}{20}$ isocaproic acid + $\frac{m}{10}$ acetic acid, equal parts	540	393

Aspartic acid combined with ammonia shows a rise of conductivity comparable with that shown by leucine and ammonia. The sum of the constituents being 672 gemmhos the mixture (ammonium aspartate) was 3170 gemmhos.

There is then no evidence from the side of the electrical conductivity to suggest a combination between amino-acids in solution. They behave towards weak bases as weak acids but not towards weak acids as weak bases. This result is moreover in agreement with those of Winkelblech and others as regards their affinity constants as acids and bases respectively, their acid-function predominating over their basic-function.

Caseinogen being a weak acid it is interesting to compare its relation to weak bases in the above manner.

Since it is insoluble in water the conductivity of a suspension of the strength of 4·54 % was determined and found to be 125 gemmhos at 11·7° C. The conductivity of a solution of the same concentration in the equivalent amount of ammonia to make a salt neutral to phenolphthalein, according to Lacqueur and Sackur, was 1732 gemmhos. The strength of the ammonia solution was $\frac{1}{25}$th normal. A similar solution was made with sodium hydrate. The results are given below :

Constituents	Conductivity in gemmhos	
	Sum	Mixture
Caseinogen + NH_4OH	312	1732
Caseinogen + NaOH	7631	1360

It was expected that the ammonium salt would exceed the sum of its constituents, but it is somewhat surprising to find that its conductivity is actually higher than that of the sodium salt.

When these two salts were subjected to the action of trypsin it was found that in four days the conductivity of the ammonium salt increased by 4050 gemmhos, whereas that of the sodium salt only increased by 1985 gemmhos. It would be advisable to test by the nitrogen content of the tannin filtrates in the two cases whether this difference is merely due to the conductivity of the ammonium salts of the products being greater than that of their sodium salts, or whether the ammonium salt of caseinogen is the more readily attacked by trypsin.

I venture to think that this peculiarity of dissociation of salts of weak acids and weak bases deserves to be taken more into consideration than it has hitherto been. For example, if the pharmacological action of strychnine is due to the kation it would seem that the acetate should be more active than the sulphate. In fact, if the action of any ions which do not form strong bases or strong acids is desired, their salts with weak acids or bases respectively should be more effective than either the free base or acid or its salts with strong acids or bases.

3. *Carbamino-acids.* Siegfried[1] has shown that when carbon dioxide is passed into a mixture of equimolecular solutions of glycocol and barium hydroxide the solution remains clear owing to the formation of a salt of a carbamino-acid. Further[2], these acids are formed when carbon dioxide is passed through solutions of free glycocol or alanine. This fact being shown by the considerable rise in electrical conductivity, over and above that due to the CO_2 dissolved in the water; the carbamino-acids are di-carboxylic acids and have therefore a much higher conductivity than the mono-carboxylic mono-amino-acids.

Before becoming aware of these latter experiments of Siegfried I had myself performed similar experiments, which entirely confirm the results of this observer.

[1] *Zeitsch. f. physiol. Chem.* XLIV. p. 85. 1905.
[2] *Zeitsch. f. physiol. Chem.* XLVI. p. 410. 1905.

Exp. Distilled water at 26° C. increased in conductivity by 40·7 gemmhos on saturation with CO_2 gas at atmospheric pressure.

A 7·5 % (=molecular normal) solution of glycine under the same conditions showed an increase of 163 gemmhos or, after subtraction of the amount due to dissolved CO_2, for the carbamino-acid, 122 gemmhos. The specific conductivity of the normal solution of the carbamino-glycine was therefore 151 gemmhos, whilst that of the original glycine solution was 29. Siegfried's corresponding values are 155 and 32.

A similar experiment with alanine gave me for the normal solution of alanine 36·7 gemmhos and for the carbamino-alanine 126 gemmhos. Siegfried's values are 55 and 201. Probably my preparation, being made from the ester, was purer than his.

Siegfried does not give any experiment with leucine. I have myself had some difficulty in obtaining satisfactory readings.

With leucine which, although obtained from the ester, had been recrystallized, I was unable to find any increase on passing CO_2, beyond that due to the CO_2 itself. It was only by making use of the fresh leucine which crystallized out from the solution obtained by boiling the ester with conductivity water, that I obtained any decisive result. This leucine was rapidly filtered off, washed with alcohol and ether, dissolved in conductivity water, boiled and its conductivity measured as soon as its temperature had fallen to 26°. In 3 % solution its conductivity was 11 gemmhos. On passing CO_2, this increased to 65·8 gemmhos, 41 of this being due to CO_2 dissolved, leaving 24·8 gemmhos for the 3 % solution of carbamino-leucine. For a solution of glycine of about the same concentration, that is $\frac{m}{4}$, Siegfried gives 89.

According to my results therefore the specific conductivity of the carbamino-acids decreases progressively from glycine through alanine to leucine.

I am unable to account for the difficulty with leucine, except to suggest that this body readily takes up carbon dioxide from the atmosphere, a property which it does not seem very probable that leucine should possess.

Summary of Conclusions.

1. Changes in "viscosity" are insufficient to account for more than a small part of the increase in conductivity under the action of trypsin, less, indeed, than 5 %.

2. The diminution in viscosity has a very different time-course from that of the conductivity. The former proceeds with great rapidity at first to become stationary at a comparatively early stage in the reaction.

3. Methods of investigating enzyme-action depending on changes of physical state, such as those with Mett's tubes containing gelatin or Grützner's or Vernon's fibrin methods, although useful for comparative estimations, give but limited information on the essential chemical work of the enzyme. Gelatin loses its power of setting after about five

minutes action of 0·16 % trypsin at 40°. Fibrin after complete solution is still present as a coagulable protein.

4. The "mechanical viscosity" of a solution, as measured by its rate of flow through a narrow tube, and its "electrical viscosity," as measured by the retardation of the velocity of migration of ions contained therein, are more or less independent properties, more especially when colloidal solutions are concerned.

5. The change in "electrical viscosity" produced by bodies in a state of colloidal solution is of the same order as that produced by non-colloidal non-electrolytes, varying from 2 to 5 % diminution in conductivity for each 1 % of the non-electrolyte present. (Arrhenius.)

6. There is some evidence that inorganic adsorbed electrolytes are set free (ionized) under the action of trypsin, but in too small an amount to account for more than a small percentage of the total increase in conductivity.

7. The production of inorganic phosphates from caseinogen by trypsin is capable of accounting for some 25 % of the rise in this particular case.

8. The chief cause of the increase in conductivity under the action of trypsin is the production of "peptones" and amino-acids, so that

9. The measurement of electrical conductivity in a trypsin digest gives a reliable and convenient method of following the course of the reaction.

10. Determinations of conductivity of certain amino-acids were made. That of the mono-carboxylic mono-amino-acids, glycine, alanine and leucine, when prepared from their ethyl-esters, is extremely low; whereas that of the di-carboxylic acids, aspartic and glutamic, is fairly high, as is also that of the di-amino-acid, lysine.

11. Although amino-acids are much more soluble in solutions of other amino-acids than in water, there is no evidence of any combination of the nature of salt-formation. In fact the mono-amino-acids of the mono-carboxylic series do not apparently act as bases towards weak acids, although they act as acids towards weak bases. The di-amino-acids on the other hand, as is well known, form salts with weak acids.

12. The formation of free carbamino-glycine and carbamino-alanine (Siegfried) is confirmed, and that of carbamino-leucine shown to take place.

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